Received: 4 September 2020 | Revised: 9 February 2021 | Accepted: 19 March 2021

DOI: 10.1111/jfpp.15482

ORIGINAL ARTICLE

Journal of Food Processing and Preservation | Institute of Food Science + Technology | ifst | WILEY

Development of extracts obtained from yerba mate leaves with different industrial processing steps: Antimicrobial capacity, antioxidant properties, and induced damage

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Funding information
Instituto Nacional de la Yerba Mate, Grant/Award Number: 310/2017; Universidad de Buenos Aires, Grant/Award Number: 2017-2020 N° 20020160100056BA Project; Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Grant/Award Number: 2015-0401

Abstract

This study was aimed at developing a yerba mate extract (YME) intended to be used as food additive with antioxidant and antimicrobial capacity. For the extraction, yerba mate (*Ilex paraguariensis*, *St Hill*) leaves collected from three industrial processing steps (*green*, *unaged-canchada*, and *aged-canchada*) were used. The *aged-canchada* YME exhibited higher theobromine, chlorogenic acid, caffeic acid, caffeine, and kaempferol than *green* YME. It was selected for further analyses as it showed the highest polyphenol content (PC = 3.7 ± 0.3 mg GAE/ml) and total antioxidant activity (TAA) by DDPH (573 ± 4 mg Trolox Eq/ml) while possessed the lowest minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations in culture media against *E. coli* and *S.* Enteritidis. Additionally, its MBC value was validated in a green juice. Moreover, *aged-canchada* YME addition (0.04%wt/vol) doubled PC, TAA_{DPPH}, and TAA_{ABTS} in the juice blend. Transmission electron microscopy images of *E. coli* cells exposed to the *aged-canchada* extract revealed severe cell damage encompassing direct rupture of outer structures with efflux of inner cell content, formation of vacuoles, vesicles, and swollen cell walls. In conclusion, the *aged-canchada* YME may be used as a food additive with the main purpose of increasing the concentration of bioactive compounds and contributing to the overall antimicrobial activity in the food product.

Practical applications

There is an increasing demand for the use of plant-based antimicrobials and/or antioxidant properties as a total or partial replacement of synthetic preservatives which are usually related to the appearance of allergies and other health issues. The present work investigated the potential use of an *aged-canchada* yerba mate extract, obtained by ultrasound, as a food additive. This extract exhibited the highest antimicrobial activity against relevant bacteria compared to other extracts obtained from different industrial stages. The extract added to a green juice doubled its concentration of antioxidants, thus positively contributing to the daily intake of these health-promoting compounds.

1 | INTRODUCTION

There is a growing tendency to reject the use of synthetic preservatives as they are highly associated with certain health issues such as allergies (Caleja et al., 2016). The use of antimicrobial natural agents represents a good alternative for obtaining safe food products which additionally may be designed with greater diversity of flavors to attract more consumer's attention. There exists today a global consumer-driven trend toward the revalorization of different teas and similar plant-based drinks, mainly due to the scientifically proven health-promoting properties of this type of beverages. In particular, the *maté* tea, a hot infusion elaborated with industrially processed yerba mate (*Ilex paraguariensis*, *St Hill*) leaves, is widely consumed as a social beverage in some Latin American countries. Multiple beneficial properties to the human health have been linked to *maté* tea consumption, including antioxidant and antimicrobial properties (Fayad et al., 2020). Moreover, there is a growing interest in manufacturing leafy green-based juices due to their health-promoting and low-calorie attributes (Biancaniello et al., 2018). Therefore, in this type of product, the use of yerba mate leaves as a plant-based additive has great potential and it also meets the expectations of those adventurous consumers interested in exploring bolder and novel flavors.

Traditional extraction methods are very time-consuming, expensive, and usually involve the use of high temperatures. Therefore, there is an increasing demand for developing new methods for the extraction of bioactive compounds from plants (Fenoglio et al., 2021). These methods should involve shorter extraction time, less organic solvent consumption, and higher prevention of pollution, compared to the traditional ones (Ke, 2015). In particular, high-intensity ultrasound has been used to improve the conventional food processing unit operations by reducing energy and chemical requirements, thus offering a more environmentally friendly option (Soquetta et al., 2019). During sonication, a phenomenon called "cavitation" takes place, in which micro bubbles of gas and/or vapor formed within a liquid, undergo violent collapse during the compression cycle of the wave, releasing large amounts of energy (Guerrero et al., 2017). This phenomenon creates hotspots, which can dramatically improve the solvent penetration into cellular materials, and even may provoke the breakdown of cells, thus releasing their biological content (Picó, 2013).

The industrial processing of yerba mate involves blanching, drying, grinding, classification, and seasoning operations (Valerga et al., 2012). Harvested yerba mate (*green*) leaves are subjected to blanching, operation that is frequently carried out in the yerba mate manufacturing facilities in a rotary drier with co-current flow. Blanching is followed by a pre-drying cycle and other drying steps by exposure to hot air until moisture content of 3% is attained. In each case, cross-flow driers are used with a bed of material of about a meter high. Inlet air temperature is about 110°C and exit air temperatures range from 50°C to 60°C, in the first drying stage and, from 70°C to 80°C, in the second one. The overall drying process typically takes from 12 to 14 hr (Isolabella et al., 2010). Once dried, leaves are coarsely ground to facilitate handling, being yerba mate usually denominated after this stage "*canchada*." Finally, the yerba mate leaves are subsequently subjected to an aging process, for the product to acquire the adequate flavor, aroma, and color, all quality aspects which are highly appreciated by consumers (Valerga et al., 2012). The plant material at this stage is frequently denominated "*aged-canchada*" yerba mate. Despite the fact that several studies focused their research on the antimicrobial properties of different commercial brands of yerba mate (Burris et al., 2015; Fayad et al., 2020; Prado Martin et al., 2013), no studies have been reported on the influence of different industrialization steps on the antimicrobial properties of yerba mate. Moreover, there are no studies revealing the type of structural damage on the microbial cells exposed to yerba mate extracts (YMEs). In particular, there is also no information comparing the structural cell damage induced by YMEs obtained by the traditional procedure involving high temperatures and those obtained by ultrasound-assisted extraction.

In the present work, the antimicrobial activity, the content of relevant bioactive compounds, and the antioxidant activity of three YMEs derived from plant material obtained at three different industrial processing steps were evaluated in culture media. The YME with the lowest MIC and highest polyphenol content was studied in a juice blend. Additionally, the induced damage on microbial cells was evaluated by transmission electron microscopy (TEM).

2 | MATERIALS AND METHODS

2.1 | Juice elaboration

A cucumber, celery, and spinach (CCS) juice blend was prepared. For the CCS juice blend obtention, cucumber and celery were thoroughly washed with water while the cucumbers were rinsed with 0.02% sodium hypochlorite and sterile water to eliminate surface microbial load and gently dried with a sterile cloth. CCS juices were obtained separately in a household juicer (Black and Decker, JE 1500, China), filtered with a muslin cloth and mixed (8:2:3). The resulting juice blend was thermally treated (80°C, 13 min) using a double jacket coil tube connected to a thermostatically controlled water bath (HAAKE, Mess-Technik, Karlsruhe, Baden-Württemberg, Germany) in order to reduce the background flora to undetectable levels (García Carrillo et al., 2017).

2.2 | Raw material

Yerba mate (*Ilex paraguariensis*) leaves collected from three industrial processing steps were used: (a) *green* yerba mate, corresponding to harvested leaves subjected to household microwave blanching (190 W, 2 min) in order to prevent enzymatic browning; (b) *unaged canchada* yerba mate (blanched and dried yerba mate leaves); and (c) *aged-canchada* yerba mate (blanched and dried leaves), additionally aged for a period of 5 months, according to the procedure described by Holowaty et al. (2016). All yerba mate samples were gently provided by *La Cachuera* industrial establishment (Apóstoles, Misiones, Argentina).

2.3 | Yerba mate extracts

Yerba mate leaves (20 g) were coarsely ground, suspended in ethanol (200 ml) and twice-sonicated (750 W, 20 kHz, 95.2 μm, 25°C, 10 min) using a high-intensity ultrasonicator provided with a 13-mm probe (Vibra-cell, Sonic Materials Inc., Chicago, Il, USA) in a double-wall cylindrical vessel (600 ml) connected to a thermostatically controlled water bath (HAAKE, Model Rotovisco RV12, Germany). The extracts were filtered (pore: 0.45 μm), evaporated (50°C, 40 min) using a rotary evaporator (Senco, Shanghai, China), resuspended in water (50 ml), and lyophilized (Stokes model 21, Philadelphia, USA) during 24 hr as stated in Ferrario et al. (2018).

Two additional methods of extraction of yerba mate leaves were also performed for the comparison purposes. The first one consisted of boiling 20 g of coarsely ground *aged-canchada* yerba mate leaves in 100 ml of distilled water during 5 min. The second one involved two consecutive stirring-based extractions at room temperature; conducted by stirring 20 g of coarsely ground *aged-canchada* yerba mate leaves in 100 ml of ethanol at 25°C during 2 hr. In both cases, the extracts were membrane filtered (pore: 0.45 μm) and lyophilized following the same procedure described above.

2.4 | Strains and preparation of inocula

The experiments were performed using *Escherichia coli* ATCC 35218, *Listeria innocua* ATCC 33090, *Salmonella* Enteritidis MA44, and *Saccharomyces cerevisiae* KE 162 (all strains were generously provided by Medica-Tec SRL, Buenos Aires, Argentina). *L. innocua* ATCC 33090 was used as nonpathogenic surrogate of *L. monocytogenes* because of its high-phenotypic similarity (Fairchild & Foegeding, 1993). Initially *E. coli*, *L. innocua*, *S.* Enteritidis, and *S. cerevisiae* inocula were prepared as described by Ferrario et al. (2015), using Trypticase Soy Agar plus 0.6% wt/wt Yeast Extract (TSAYE) and Sabouraud Dextrose agar (SAB), for bacteria and *S. cerevisiae*, respectively. All inocula were harvested by centrifugation (1,475 g, 5 min) (Labnet International Inc., Edison, NJ, USA), washed twice with saline solution, and resuspended in peptone water (0.1% wt/vol) to give a cell density of 10^7–10^9 CFU/ml.

2.5 | Minimum inhibitory (MIC) and bactericidal (MBC) concentrations in culture media

Different concentrations of the freeze-dried YMEs, obtained according to the optimized extraction procedure were added to SAB or TSAYE broths (pH = 7.0, 10 ml) to achieve a PC range of 2.2–3.6 mgGAE/ml. The resulting broths were inoculated with 100 μl of *E. coli*, *S.* Enteritidis, *L. innocua*, or *S. cerevisiae* and incubated under agitation at 37°C or 27°C in the case of bacteria or the yeast, respectively. Samples were taken at regular intervals for the analysis of survivors during 7-day storage. Microbial populations were determined using a spiral plater (Autoplate 4000, Spiral Biotech, USA). Plates were incubated for 72 hr at 37°C (bacteria) and 27°C (yeast). A counting grid was used for the enumeration of colonies. Survival curves were generated from the experimental data by plotting log *N* (where *N* is the number of CFU/mL at a given time) versus time of treatment. Inoculated broths without YME addition were used as controls. For each condition, the MIC and the MBC were determined after 1, 5, or 7 days of system storage. The MIC value was determined as the lowest concentration tested which prevented from the microbial growth (inhibition) compared to the original inocula (10^7 CFU/ml). Whereas, the MBC value was determined as the lowest concentration tested to which the bacterial death (inactivation) was observed. The experiments were run in triplicate.

2.6 | Total polyphenol content

The total polyphenol content (PC) in the extracts was determined using the method described by Ferrario et al. (2018). Samples (0.5 ml) were mixed with 2.5 ml of Folin–Ciocalteu phenol reagent (10% vol/vol) and followed by addition of Na_2CO_3 (7.5% wt/vol, 2 ml). The reactive mixture was allowed to stand for 3 hr in darkness, and the formation of blue color, as indicator of PC, was quantified in 100-fold diluted samples at 740 nm using a UV-VIS spectrophotometer (V-630, Jasco, Tokyo, Japan). PC values were derived from the calibration curve ($y = 13.2465x + 0.0518$, $R^2 = 0.99$) and expressed as mg of Gallic acid equivalents per milliliter of the sample (mg GAE/ml).

2.7 | In vitro antioxidant activity evaluation

2.7.1 | DPPH radical scavenging assay

The antioxidant activity (TAA) was evaluated by a colorimetric method based on the measure of the free radical-scavenging capacity of the sample with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical (Merck, Billerica, USA), according to the methodology proposed by Ferrario et al. (2018). Eighty (80) microliter of 1500-times diluted samples in methanol were mixed with DPPH (0.02 g/L in methanol, 2,200 μl). The reaction mixture was led to stand in the dark (30 min at 25°C) and the corresponding absorbance was measured at 515 nm using a UV-VIS spectrophotometer (V-630, Jasco, Tokyo, Japan). TAA values were determined from the calibration curve ($y = 0.00084x - 0.0024$, $R^2 = 0.99$), and expressed as Trolox equivalents (mg Trolox Eq/ml).

2.7.2 | ABTS radical scavenging assay

This assay is based on the ability of substances of scavenging the 2-2′-azino-bis ethylbenzthiazoline-6-sulfonic-acid ($ABTS^+$) radical cation, according to the procedure reported by Shalaby and Shanab (2013). The radical cation was prepared by mixing 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate (1/1, vol/vol), and leaving the reactive mixture for 18 hr, when a steady absorbance

value was obtained. The $ABTS^+$ solution was diluted with pure ethanol (1/10, vol/vol). The photometric assay was conducted using 8 ml of the $ABTS^+$ solution vigorously mixed with 0.2 ml of the tested samples (1/1500, vol/vol), and measured at 734 nm after 1.5 hr of contact time. The results were determined from the calibration curve ($y = 0.0031x + 0.0264$, $R^2 = 0.9901$), and expressed as Trolox equivalents (mg Trolox Eq/ml).

The scavenging capacity for DPPH and ABTS methods was calculated as:

$$\%\,\text{Inhibition} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}}\right) \times 100 \quad (1)$$

2.8 | High-pressure liquid chromatography (HPLC)

Chromatographic analysis was performed as described by Deladino et al. (2013) with slight modifications. Briefly, a 1525 HPLC system (Waters, USA) equipped with a binary pump and the 2996 Photodiode Array (PDA) detector (Waters, USA) was used. A Restek Roc C18 (250 × 4.6 mm) column was used. The mobile phase A was composed of acetonitrile while mobile phase B consisted of water and phosphoric acid (99:1). The staggered gradient program was optimized by the percentage change of mobile phases and set as follows: T_{time}(min)/mobile phase-A : B (%): T0/5:95, T5/5:95, T15/20:80, T30/20:80, T35/30:70, T65/30:100, and T73/5:95 at a flow rate of 1 ml/min.

The powdered samples (50 mg) were diluted in 2 ml HPLC water, filtered through a 0.22 μm nylon membrane, and injected (20 μl) onto the column (LiChrospher® 100 RP-18e (250 mm × 4 mm, 5 μm) which was kept at 30 ± 0.5°C. The identification of the selected compounds was performed by co-injection of the rutin, caffeine, chlorogenic acid, and theobromine standards (all reagents from Sigma-Aldrich, St. Louis, MO, USA) and subsequent comparison of retention times and characteristic absorption spectra. Quantification was performed using the external standard technique. The area under each identified peak of the chromatogram was determined and compared with the corresponding standards, integrating the wavelength of maximum absorption for each compound which was 271.5 nm for theobromine and caffeine, 325.1 nm for chlorogenic acid, and 356.3 nm for rutin. The results were expressed as mean ± standard error in mg/g dried extract of three replicates.

2.9 | TEM sample preparation and analysis

E. coli cells were used to investigate the structural damage induced by *aged-canchada* YME. The *E. coli* cells were incubated in TSB (control) or in TSB plus *aged-canchada* YME for 1 day (37 ± 1°C). For comparison purposes cells exposed to an YME obtained by the traditional extraction were evaluated ($YME_{traditional}$). Cells were centrifuged (1,475 g, 5 min) and the supernatant was discarded. The pellet was resuspended in 2 ml of glutaraldehyde (2.5 g/100 ml of 0.1 M phosphate buffer) and stand for 2 hr at 4°C. Then, it was washed and resuspended with 0.1 M of phosphate buffer and post-fixed with $KMnO_4$ (2 g/100 ml) in 0.1 M of phosphate buffer for 17 hr at 4°C; washed with distilled water, dehydrated with alcohol series, and embedded in Epon 812 (Shell Chemical Company, USA) during 48 hr (Bolondi et al., 1995). Ultrathin sections were examined with an Opton Electron Microscope EM 109 (Lanais-Mie, UBA-CONICET, Buenos Aires, Argentina). Untreated cells and cells incubated with $YME_{traditional}$ served as negative and positive controls, respectively.

2.10 | Antimicrobial activity and physicochemical characterization of the CCS juice blend loaded with an *aged*-canchada YME

The MBC values determined in culture media corresponding to the *aged-canchada* YME were tested in a fresh squeezed CCS juice blend (pH: 5.7 ± 0.1, 3.1 ± 0.2 °Brix, 7,411 ± 11 NTU) against *E. coli* ATCC 35218 and *S.* Enteritidis MA 44.

The CCS juice samples with or without *aged-canchada* YME were inoculated with 100 μl of *E. coli* or *S.* Enteritidis and analyzed for survivors at predetermined intervals between 0 and 5 days, as described in the Section *MIC and MBC in culture media*. A lower concentration of *aged-canchada* YME was selected for the physicochemical characterization of the juice blend (4 mg/ml) compared to the obtained MBC value. This concentration had been well accepted by consumers in a sensory test of a carrot–orange juice blend loaded with YME (Ferrario et al., 2018). Additionally, CCS juice samples added or not with *aged-canchada* YME were characterized by PC, TAA_{DPPH}, TAA_{ABTS}, and FC content, as described above.

2.11 | Statistical analysis

Multivariate analysis of variance (MANOVA) was applied to determine differences in the concentrations of PC, TAA, FC, caffeine, chlorogenic acid, caffeic acid, theobromine, and kaempferol in the extracts. In case of finding significant differences, Hotelling with Bonferroni corrections test was used. Principal Component Analysis (PCA) was applied to illustrate the spatial relationship among the three obtained YMEs and PC, TAA, caffeine, chlorogenic acid, caffeic acid, theobromine, and kaempferol. Statistical analyses were conducted using InfoStat 2009 (InfoStat Group, FCA-UNC, Córdoba, Argentina).

3 | RESULTS AND DISCUSSION

3.1 | Characterization of the YM extracts

3.1.1 | Total antioxidant activity and total PC

The PC values corresponding to the extracts obtained from YM leaves at three different industrial processing steps (*aged-canchada*,

unaged-canchada, and *green*) are displayed in Figure 1a. *Aged-canchada* YME exhibited the highest PC value (3.7 ± 0.3 mg GAE/ml or 6.1 ± 0.4 mg GAE/g dried-YME), followed by *unaged-canchada* YME (1.9 ± 0.0 mg GAE/ml or 3.1 ± 0.0 mg GAE/g dried-YME), and *green* YME (1.5 ± 0.1 mg GAE/ml or 2.6 ± 0.1 mg GAE/g dried-YME) ($p < .001$).

Several extraction methods have been reported in literature, encompassing a wide range of PC values. For instance, Turkmen et al. (2007) obtained similar (0.50 to 2.10 mg GAE/g) PC values to the ones reported in the present work by stirring 0.2 g of black tea (*Camellia sinensis L*) samples in 10-ml absolute ethanol at 23°C, but for a much longer extraction period (2–18 hr). In contrast, Prado Martin et al., 2013) reported that a hydroethanolic (40:60) extract obtained by percolation of yerba mate exhibited a lower PC value (0.19 mg GAE/g). In the same trend, Bassani et al. (2014) reported low PC values, (0.34 to 0.48 mg GAE/mL) in an extract obtained by heating 2 g of ground roasted yerba mate leaves in distilled water (100 mL) at 60-90 °C during 5-10 min.

Figure 1b illustrates TAA determined by both, DPPH and ABTS assays, corresponding to the extracts obtained from yerba mate leaves with different industrial processing steps. As it was observed for PC, the YME from *aged-canchada* leaves exhibited the highest TAA value (573.6 ± 3.4 mg Trolox Eq/ml or 81.9% inhibition) determined by DPPH, followed by *unaged-canchada* yerba mate (420.6 ± 54.2 mg Trolox Eq/ml or 72.0% inhibition) and *green* yerba mate (302.6 ± 3.4 mg Trolox Eq/ml or 60.3% inhibition). When TAA was determined by the ABTS assay, the YME obtained from *green* yerba mate displayed the highest TAA value (1,160 ± 230 mg

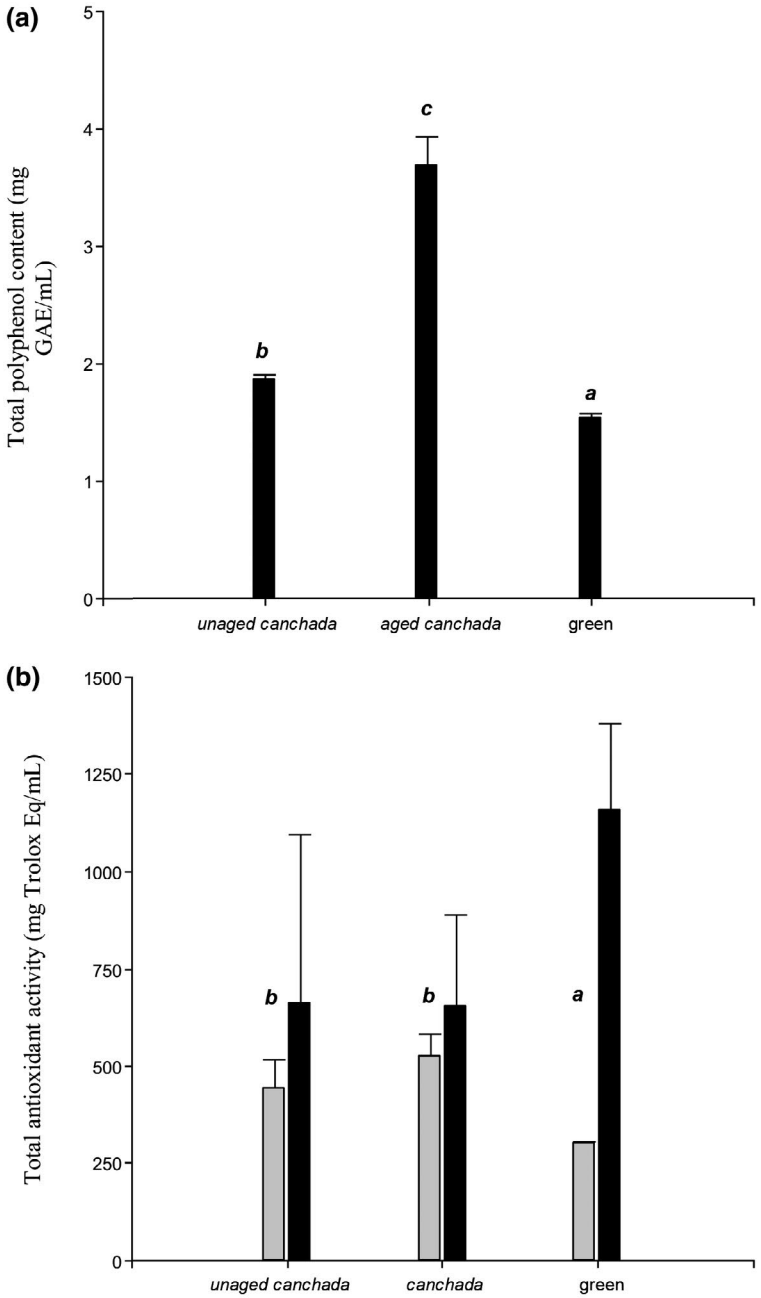

FIGURE 1 Total polyphenol content (PC, mg GAE/ml) (a), total antioxidant activity (mg Trolox Eq/ml) by DPPH (▒) or ABTS (■) (b), of *unaged-canchada*, *aged-canchada*, and *green* YMEs. Different letters above the bars indicate statistically significant differences ($p < .0001$) by two-way MANOVA. Standard deviation (I)

TABLE 1 Quantification of theobromine, caffeine, kaempferol, chlorogenic acid, and caffeic acid by HPLC in the extracts obtained from yerba mate leaves corresponding to different industrial stages

	Aged-canchada YM (mg/g dried extract)	Unaged-canchada YM	Green YM
Theobromine	7.61 ± 1.26	10.21 ± 0.46	3.10 ± 0.31
Chlorogenic acid	54.86 ± 8.18	57.64 ± 2.41	39.33 ± 3.92
Caffeic acid	40.45 ± 5.64	44.27 ± 1.54	32.50 ± 2.15
Caffeine	60.64 ± 7.59	65.55 ± 2.05	39.95 ± 4.40
Kaempferol	11.59 ± 1.83	12.31 ± 1.54	10.27 ± 1.23

Trolox Eq/ml), followed by *unaged-canchada* YME (650 ± 440 mg Trolox Eq/ml) and *aged-canchada* YME (660 ± 240 g Trolox Eq/ml) extracts (Figure 1b). In agreement, Bassani et al. (2014) obtained similar inhibition percentages (52.4%–80.7%) by DPPH after heating grounded roasted yerba mate leaves (brand: Leão Junior®, place of production: Paraná, Brazil) in water at 60–90°C for 5–10 min. In contrast, Turkmen et al. (2007), obtained inhibition percentages around 5%–6% by DPPH after stirring for 2–18 hr yerba mate leaves in pure ethanol. Nevertheless, these authors reported up to 94% inhibition by DPPH when a hydroethanolic solvent (50:50) was used for 3 hr.

An increase in the PC and TAA by DPPH values was observed in the extracts obtained from *aged-* and *unaged-canchada* yerba mate leaves compared to the *green* sample. Therefore, the industrial processing conditions involving high temperature and high relative humidity, during drying and aging of the yerba mate leaves, may have induced a higher release of polyphenols and antioxidant compounds during the extraction procedure compared to the *green* samples.

With regards to the ABTS or DPPH assays, it is important to highlight that in the present work, TAA values of a given YM sample showed differences between one estimate and the other. ABTS assay is a useful tool to determine the antioxidant activity of both, lipophilic and hydrophilic antioxidants in various matrices such as body fluids and herbal extracts. Nevertheless, reactions are slowed by the presence of big molecules or high concentrations of rings and multiple-OH; hydrogen atom transfer and bulky adducts. ABTS is reduced by single phenols and small molecules. As the size and structural complexity of the phenol increases, the reaction becomes increasingly impaired (Schaich et al., 2015). Likewise, interactions between antioxidants and DPPH are also determined by the antioxidant's structural conformation. Some compounds react immediately with DPPH, but some others, with complex structures impede access to DPPH at low concentrations and they strongly block reaction at high concentrations (Martysiak-Żurowska & Wenta, 2012). In contrast to the ABTS assay, DPPH runs in methanol; and therefore, electron transfer dominates upon hydrogen atom transfer required to dissolve DPPH (Schaich et al., 2015). Outstandingly, in this study, higher TAA values were detected by DPPH in the YMEs obtained from dried YM leaves (*unaged and aged-canchada*) compared to the ABTS assay. In contrast, the extract obtained from *green* yerba mate leaves displayed higher TAA content by ABTS compared to DPPH. This could be attributed to the fact that *green* YM samples exhibit high water content thus, impeding DPPH to detect certain antioxidants present in these samples, probably due to the non-polarity of the methanol used as reaction solvent.

The ultrasound-assisted extraction resulted in a fast and more efficient method for the recovery of polyphenol and total antioxidant compounds from yerba mate leaves as it yielded high PC and TAA values (PC = 3.7 ± 0.3 mg GAE/ml, TAA by DPPH = 573.6 ± 3.4 mg Trolox Eq/ml, TAA by ABTS = 1,160 ± 230 mg Trolox Eq/ml), at milder conditions (25°C). Additionally, a relevant aspect to be considered for its industrial application is the reduction of the energy requirements, thus contributing to decrease both, environmental and the economic costs. Even though the traditional extraction (100°C, 5 min) yielded the highest value of PC (4.9 ± 0.6 mg GAE/ml), the high temperature employed could seriously alter the activity of the bioactive compounds in the extract (Khan et al., 2018). Regarding the stirring-based extraction method performed at room temperature, the lowest PC value (1.42 mg GAE/ml) was determined. In this respect, Anusic (2012) evaluated PC in a YME obtained by two cycles of stirring (3 hr plus 1 hr) in methanol at room temperature, and reported a PC value of 0.1 mg GAE/ml.

3.1.2 | HPLC quantification of theobromine, chlorogenic acid, caffeic acid, caffeine, and kaempferol

All the YM extracts assayed in this study were characterized in terms of the concentrations of those polyphenols mostly recognized as health-promoting compounds (Anesini et al., 2012): theobromine, chlorogenic acid, caffeic acid, caffeine, and kaempferol (Table 1). The elution chromatogram corresponding to the *green* YME is shown in Figure 2. Five major peaks were identified as theobromine (t_r = 12.7 min), chlorogenic acid (t_r = 16.0 min), caffeic acid (t_r = 18.2 min), caffeine (t_r = 18.9 min), and kaempferol (t_r = 38.5 min). All the extracts obtained in this study from YM leaves at different industrial processing steps showed the same profile, only differing in the area of the peaks. The highest concentration of the five major peaks were observed for the *unaged-canchada* YME: theobromine 10.21 ± 0.46 mg/g, chlorogenic acid 57.64 ± 2.41 mg/g, caffeic acid 44.27 ± 1.54 mg/g, caffeine 65.55 ± 2.05 mg/g, and kaempferol 12.31 ± 1.54 mg/g.

Literature reports a wide concentration range of these biocompounds determined in YMEs obtained by different extraction procedures. According to this study, the ultrasound-assisted extraction was more effective in the obtention of these phytochemicals compared

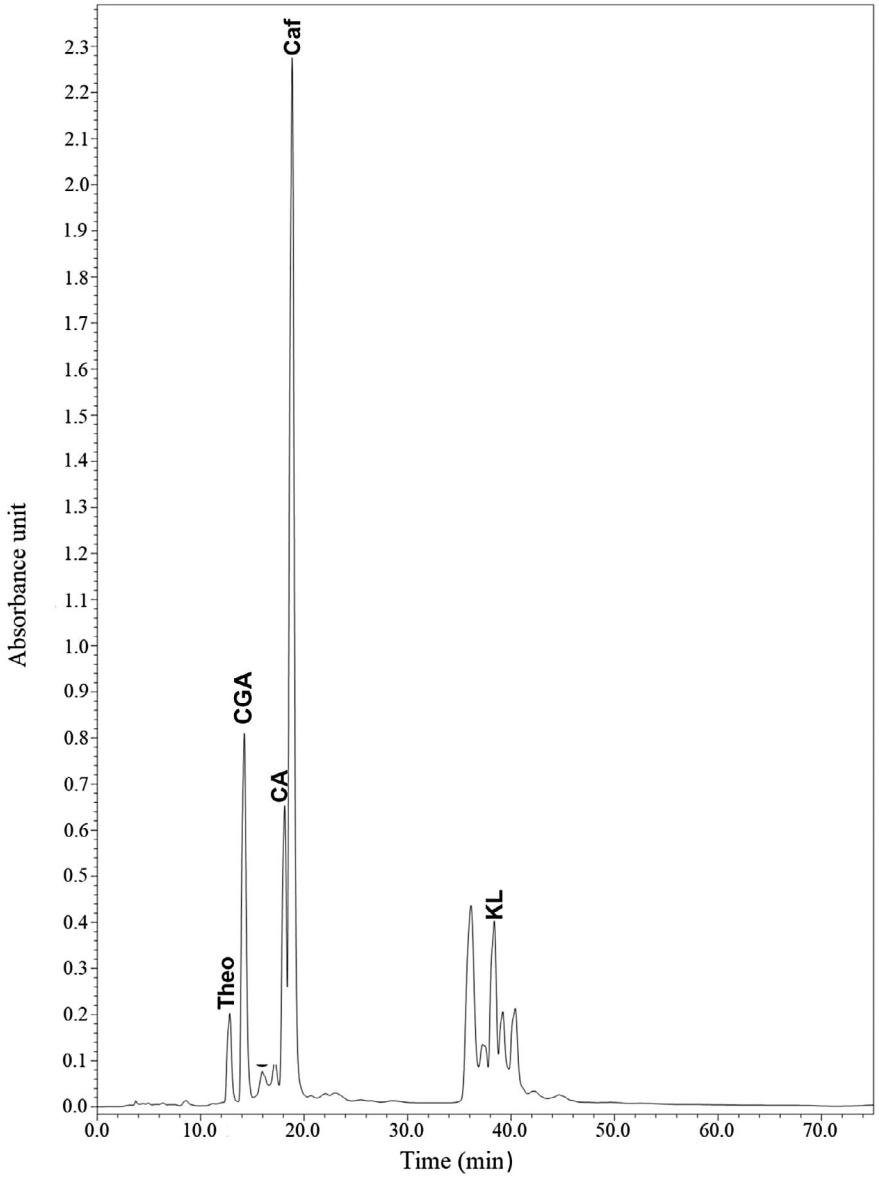

FIGURE 2 HPLC chromatogram obtained at 271 nm corresponding to the green yerba mate extract. The identified compounds were the following: theobromine (Theo), chlorogenic acid (CGA), caffeic acid (CA), caffeine (Caf), and kaempferol (KL)

to the traditional methods. In particular, Anesini et al. (2012) evaluated by HPLC the content of caffeine, chlorogenic acid and caffeic acid of an aqueous YME obtained by heat (20 min at 100°C). They determined lower concentration values of these three compounds (chlorogenic acid: 20.7 mg/g, caffeic acid: 0.033 mg/g, and caffeine content: 13.5 mg/g) compared to the present study. In the same trend, Isolabella et al. (2010) quantified caffeine, theobromine, caffeic, and chlorogenic acids by HPLC in YME obtained by immersion of yerba mate leaves in boiling water during 20 min. They reported lower caffeic acid (0.3 mg/g), caffeine (15 mg/g), and chlorogenic acid (20 mg/g) content compared to the results obtained in the present work, but similar amount of theobromine (5 mg/g). Bojić et al. (2013) reported lower concentrations of chlorogenic acid (3.0 mg/g), caffeine (5.4 mg/g), and theobromine (2.7 mg/g) in a liquid YM extract obtained by immersion of yerba mate leaves in boiling during 30 min compared to the values obtained in the present study. Additionally, they did not detect caffeic acid and kaempferol. Moreover, Scipioni et al. (2007) determined the caffeine content in extracts obtained by soaking YM leaves of different brands in hot water (90°C, 1 hr) followed by filtration. They reported lower caffeine values (4.9–12.0 μg/g) compared to those obtained in the present study.

3.2 | MIC and MBC testing of the YMEs

The extracts obtained from *aged-canchada*, *unaged-canchada*, and *green* yerba mate leaves were used in a microbial challenge study in culture broth. Figure 3 illustrates *E. coli* ATCC 35218, *Listeria innocua* ATCC 33090, *Salmonella* Enteritidis MA 44, and *Saccharomyces cerevisiae* KE 162 evolution in culture broth added with YM extracts from green, *aged-*, and *unaged-canchada* YM leaves during storage.

Untreated systems were able to grow reaching up to 10^8–10^{10} CFU/ml by day 1 of storage (data not shown). All the YM extracts effectively inhibited growth or even inactivated all assayed bacteria in the culture broth (Figure 3a–c). In particular, MIC values of 2.2 mg GAE/mL (caffeine: 9.4 μg/ml), 3.2 mg GAE/ml (caffeine: 13.5 μg/ml), and 2.47 mg

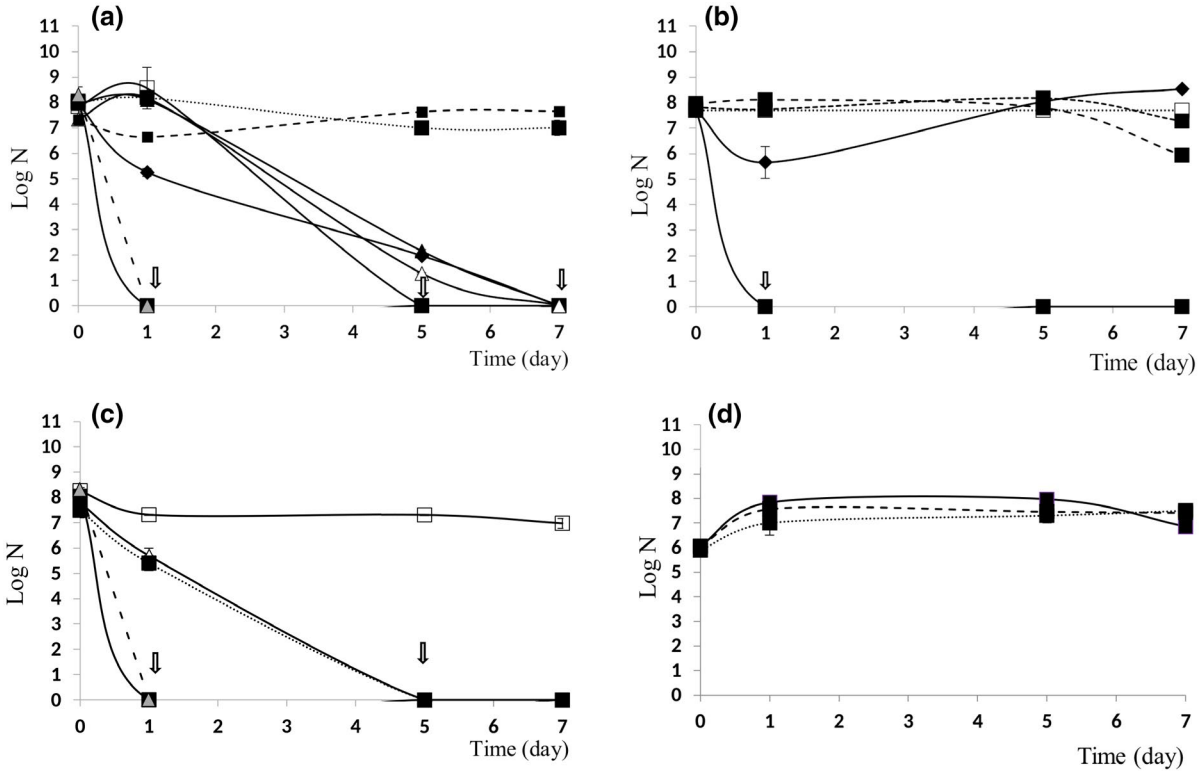

FIGURE 3 Evolution of *E. coli* ATCC 35218 (a), *L. innocua* ATCC 33090 (b), *S.* Enteritidis MA 44 (c), or *S. cerevisiae* KE 162 (d) in culture broth added or not (control) with the developed YMEs from leaves collected at different industrial processing steps, and stored during 7 days (27 or 37°C) under agitation. *Aged-canchada* YME: 2.2 mgGAE/ml (□,—), 2.5 mg GAE/ml (□,- - -), 2.7 mg GAE/ml (△,—), 3.2 mg GAE/ml (♦,—), and 3.6 mg GAE/ml (■,—); *unaged-canchada* YME: 3.6 mg GAE/ml (■,- -); *green* YME: 3.6 mg GAE/ml (■, · · ·), cucumber–celery–spinach (CCS) juice plus 3.6 mg GAE/ml *aged-canchada* YME (▲,— - — -) and standard deviation (I)

GAE/ml (caffeine: 10.6 μg/ml) corresponding to *E. coli*, *L. innocua*, and *S.* Enteritidis, respectively, were observed for *aged-canchada* YME after the first day of storage, remaining this value constant until the end of the assay (Figure 3a–c). Moreover, the same MIC value of 3.6 mg GAE/mL (caffeine: 15.3 μg/ml) was recorded for *unaged-canchada* and *green* YMEs for all bacteria along storage, indicating that *aged-canchada* YME had higher concentration of antimicrobial compounds.

Additionally, certain concentrations of the *aged-canchada* YME were able to inactivate all bacteria. For instance, MBC values of 3.6 mg GAE/ml (caffeine: 22.8 mg/g, chlorogenic acid = 20.6 mg/g, and caffeic acid = 15.2 mg/g) at the first day of storage and 2.7 mg GAE/ml (caffeine: 17.3 mg/g, chlorogenic acid = 15.7 mg/g, and caffeic acid = 11.6 mg/g) at the fifth day of storage of *aged-canchada* YME were obtained for *E. coli*, *L. innocua*, and *S.* Enteritidis, respectively (Figure 3a–c). Likewise, only in the case of *E. coli*, an important decrease in the MBC values was observed along storage, as complete inactivation was observed on the seventh day in presence of 2.2 mgGAE/mL *aged-canchada* YME (Figure 3a). Regarding the extracts obtained from other industrial steps, the concentration of 3.6 mgGAE/mL of *unaged-canchada* YME only reduced *S.* Enteritidis population from the beginning of the storage and achieved complete inactivation by day 5 (Figure 3c). *E. coli* was the most sensitive strain to *aged-canchada* YME as it showed the lowest MIC (2.2 mgGAE/ml) and MBC (3.2 mgGAE/ml) values at all assayed days. In contrast to the previous results, *S. cerevisiae* was the most resistant strain as it was not inhibited by any of the assayed extracts, thus displaying a similar growth profile than the control system (Figure 3d).

Prado Martin et al. (2013) evaluated the antimicrobial activity of samples of yerba mate collected from different commercial brands. Hydroethanolic extracts were maintained for 96 hr at different pH values (6–8) and filtered. These authors observed that *Staphylococcus aureus* ATCC 25922, *Listeria monocytogenes* ATCC 07644, and *S.* Enteritidis 13076 were inhibited at higher PC (193.9 mg GAE/g extract) concentration than the ones reported in the present work. Whereas they reported, that *E. coli* ATCC 25922 was not inhibited at any assayed condition. The higher inhibition observed in the present study may be attributed to the use of high-intensity ultrasound during the extraction procedure, which may have induced the additional release of other chemicals, thus contributing in a synergistic way to the antimicrobial activity. Similarly, Fayad et al. (2020) evaluated the MIC values of an aqueous extract of yerba mate, obtained by hot water immersion (70°C, 2 hr, 1 g leaves, 3.6 ml water) against different bacteria. They reported MIC values 1.88–3.75 mg/ml extract and 2.5–10 mg/ml extract against different strains of *Staphylococcus aureus* and *Acinetobacter baumannii*, respectively. No inhibition was found against *E. coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* at the maximum concentration assayed (30 mg/ml). Unfortunately, these authors did not evaluate the PC of the YME for

comparison purposes. Even though these authors identified relevant polyphenols such as caffeine, theobromine, and stigmasterol, among others, they did not quantify their concentrations to compare to the values obtained in the present study.

A principal component analysis (PCA) was applied to illustrate the association between the studied YMEs obtained with yerba mate leaves from different industrial processing steps with the MIC values obtained for the three bacteria, the PC, TAA determined by the DPPH and ABTS assays, caffeine, caffeic acid, chlorogenic acid, theobromine, and kaempferol (Figure 4). The two principal components (PC 1 y PC 2) explained the total variance 57.4% and 42.0%, respectively, clearly separating the evaluated YM extracts. The first axis, PC 1 contrasted PC, caffeine, caffeic acid, chlorogenic acid, theobromine, and kaempferol and TAA (DPPH) values positively, and the MIC values against the three bacteria (ABTS), negatively. The second axis (PC 2) was defined positively by PC and TAA (ABTS), and negatively by theobromine, caffeic acid, kaempferol, and the MIC values against bacteria. The *aged-canchada* YME showed the highest values of PC and TAA (DPPH) and the lowest MIC values against the three bacteria. Additionally, it displayed high values of caffeine, caffeic and chlorogenic acids, theobromine, and kaempferol along with the *unaged-canchada* YME. The *green* YM extract exhibited the lowest values of PC, TAA (DPPH), caffeine, caffeic acid, chlorogenic acid, theobromine, and kaempferol, but similar TAA (ABTS) than *aged-canchada*.

The high temperature and humidity conditions applied to *aged* and *unaged-canchada* yerba mate leaves during the drying and aging steps, may had induced the release of polyphenol compounds and antioxidants determined by DPPH. Due to the high water content of the *green* YME, certain compounds might not have been detected by DPPH determination as it is run in methanol. In contrast, the *green* YME displayed a similar TAA value compared to the *aged-canchada* YME when determined by ABTS method, as this technique detects both, polar and no polar compounds.

The extract derived from *aged-canchada* yerba mate leaves was more effective compared to the other assayed extracts with regards to the observed antimicrobial activity. This extract displayed the highest PC value, but similar caffeine, caffeic acid, and chlorogenic content than *unaged-canchada* extract. All these compounds display scientifically proven antimicrobial properties. It has been reported that caffeine concentrations of 62–2000 μg/ml inhibit a wide range of bacteria (Nonthakaew et al., 2015). While, caffeic acid (0.9–1.8 mg/ml) inhibited *E. coli* DMF 7503, *S. cerevisiae* DMF 2107, and *Listeria monocytogenes* DMF 5776 in agar (Merkl et al., 2010). Moreover, caffeic acid (0.4 mg/ml) completely inhibited *S. aureus* in chicken soup (Stojković et al., 2013). On the same trend, Özçelik et al. (2011) reported that caffeic acid (8–128 μg/ml) and chlorogenic acid (8–128 μg/ml) inhibited *E. coli*, *Candida albicans*, *Bacillus subtilis*, *and Pseudomonas aeruginosa* in culture broth.

A higher PC was recorded in the *aged-canchada* sample compared to the *unaged-canchada* one; however, higher levels of caffeine, kaempferol, caffeic acid, chlorogenic acid, and theobromine were observed in the later one. Therefore, it is possible that additional polyphenols with antimicrobial capacity may be present in the *aged-canchada* and may not have been identified.

3.3 | Morphological changes of *E. coli* cells exposed to YM extracts evaluated by TEM

TEM was used to visualize the morphological changes induced in *E. coli* cells exposed to the *aged-canchada* YME. Because of the high cost of the method, *E. coli* was selected as it showed the highest sensitivity to YME. Figure 5a,b illustrates the TEM micrographs

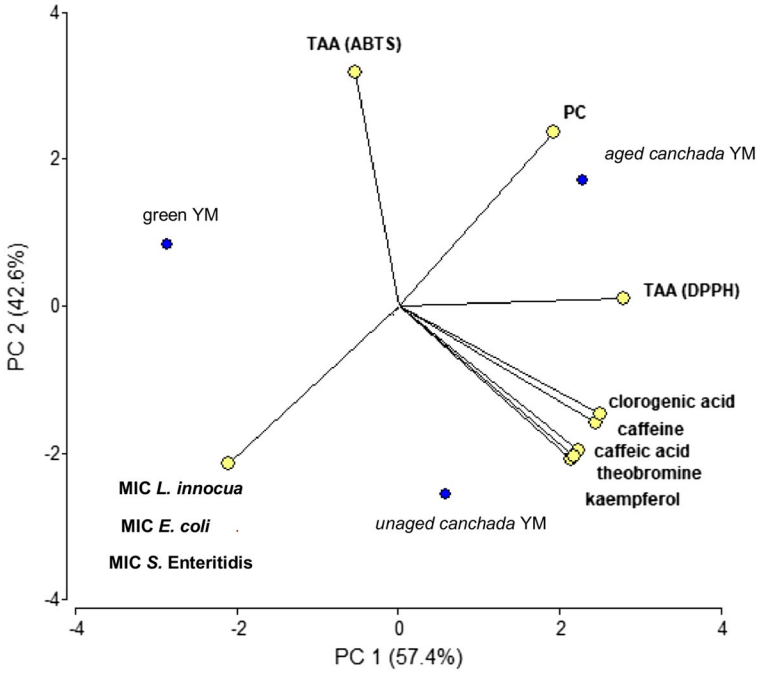

FIGURE 4 Principal component analysis (PCA) bi-plot of the MIC values against *L. innocua* ATCC 33090, *S.* Enteritidis MA 44, and *E. coli* ATCC 35218, total polyphenol content (PC), total antioxidant activity (TAA) determined by ABTS and DPPH methods, theobromine, chlorogenic acid, caffeic acid, caffeine, and kaempferol content of yerba mate extracts obtained from *green*, *unaged-canchada*, and *aged-canchada* yerba mate leaves

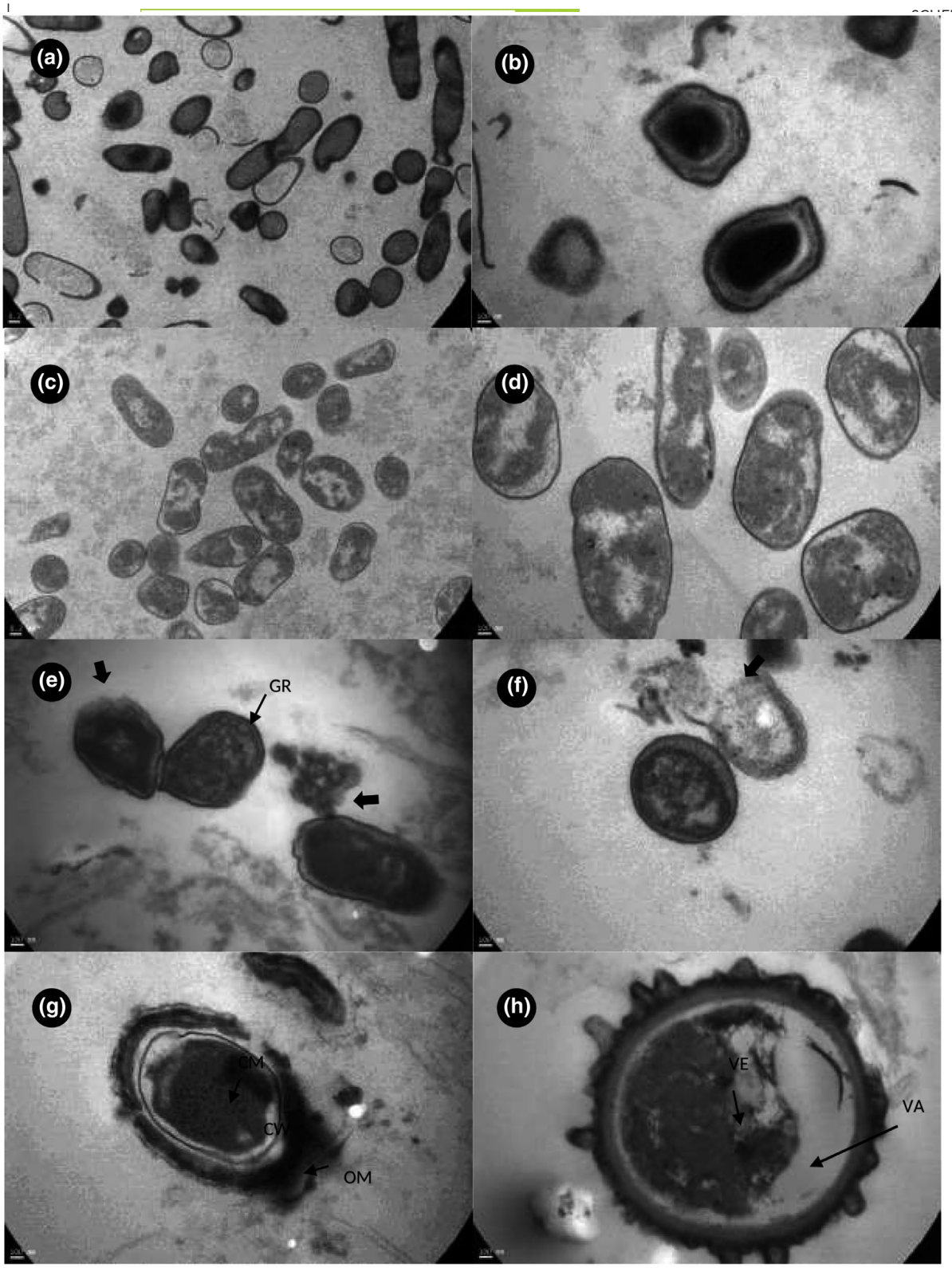

FIGURE 5 TEM images of *E. coli* ATCC 35218. (a, b): untreated cells; (c, d): cells exposed to *aged-canchada* extract (37.5%wt/vol) obtained by thermal extraction, for 1 day (37 ± 1°C); (e–h): cells exposed to ultrasound-assisted *aged-canchada* extract (37.5%wt/vol) for 1 day (37 ± 1°C); (a) group of intact cells with similar general rod-shape aspect (longitudinal section); (b) cross section of intact cells showing well defined outer membrane, cell wall, and plasmalemma with high electronic density; (c, d) general aspect (c) and magnification (d) of a group of cells with intact outer membrane and cell wall but showing coagulated lumen with low electronic density and discontinuities, cytoplasmic membrane shrank (e): cell with single puncture site of outer structures with efflux of inner material (arrow), presence of granules; (f): broken cell wall and plasmalemma with separate fragments with efflux of inner material (arrow); (g) general aspect of a cell with intact plasmalemma and shrunk lumen but disrupted cell wall and outer membrane; presence of fragments; (h) cross section of cell showing presence of vacuole and vesicle. Scale: a, c: 0.2 μm; b, d, e, f, g, and h: 100 nm. CM, cytoplasmic membrane; CW, cell wall; ED, electronic density; GR, granule; OM, outer membrane; VA, vacuole; VE, vesicle

corresponding to the untreated *E. coli* cells, which displayed the typical rod-shape, and electron-dense cytoplasm and smooth and continuous double membrane. Figure 5b, which was obtained at greater magnification, shows the well-defined outer cell membrane, cell wall, and unalterable cytoplasmic membrane with an inner content with high electronic density.

Figure 5c–h shows *E. coli* cells that were exposed to *aged-canchada* extracts obtained either by traditional thermal extraction (Figure 5c,d) or the proposed ultrasound-assisted extraction (Figure 5e–h), and incubated for 1 day at 37°C. Ultrastructural studies revealed that certain internal damage occurred for the majority of cells exposed to the YME obtained by thermal extraction in the population. The inner cell content appeared coarse, with lower electronic density, cytoplasmic membrane shrinkage, and cytoplasm discontinuities compared to the untreated cells (Figure 5c,d). However, cell outer structures remained intact without observing leakage of the intracellular compounds (Figure 5d).

Conversely, *E. coli* cells exposed to the *aged-canchada* YME (Figure 5e–h) displayed more severe structural alterations compared to the one obtained by thermal treatment. In particular, *E. coli* cells underwent lysis and showed swollen cell walls, thus resulting in leakage of intracellular material. The cytoplasm was extensively granulated and retracted from the outer membrane (Figure 5e–g). Cell walls and plasmalemma also appeared fragmented (Figure 5f,g) and/or displayed outer membrane roughening with the formation of blebs (Figure 5g,h). In addition, many cells exhibited inner vacuoles, vesicles (Figure 5h), and granules (Figure 5e) within the cytoplasm.

Therefore, more severe structural alterations were observed in *E. coli* cells exposed to the ultrasound-assisted *aged-canchada* YME compared to the YME obtained by thermal extraction, may suggest the presence of other non-identified compounds in the YME or even a higher concentration of the compounds with antimicrobial activity that may have induced damage in a synergistic way (Burris et al., 2012). The higher inactivation effectiveness of the ultrasound-assisted YME may be attributed to the cavitation process that occurred during sonication which may have caused cell wall rupture, thus enhancing solvent contact with available extractable cell material (Guerrero et al., 2017).

3.4 | Microbial validation study and physicochemical characterization of the CCS juice blend loaded with *aged-canchada* YME

The *aged-canchada* YME was selected to enrich the CCS juice blend as it displayed the highest PC (PC = 3.7 ± 0.3 mg GAE/ml) and TAA, while exhibiting the lowest MIC and MBC values against all assayed bacteria.

Figure 3 also illustrates *E. coli* and *S.* Enteritidis growth dynamics in the CCS juice blend with or without (control) *aged-canchada* YME addition along storage at abuse temperature (37°C). Microbial populations corresponding to the control juice sample remained constant throughout the whole storage (data not shown). In contrast, undetectable counts were observed for the juice samples loaded with YME (Figure 3a,c), thus confirming the results obtained in culture media. Therefore, the *aged-canchada* YME did not lose antimicrobial activity when it was incorporated to a real food matrix.

The CCS juice blend added with *aged-canchada* YME at the very low concentration of 4 mg/ml significantly doubled PC (54 ± 1 μg GAE/ml), TAA_{DPPH} (1.1 ± 0.1 mg Trolox Eq/ml), and TAA_{ABTS} (0.5 ± 0.1 mg Trolox Eq/ml) compared to the control juice samples (PC = 32 ± 1 μg GAE/ml, TAA_{DPPH} (0.4 ± 0.0 mg Trolox Eq/ml), and TAA_{ABTS} (0.2 ± 0.0 mg Trolox Eq/ml.

4 | CONCLUSIONS

The ultrasound-assisted extraction procedure used in this study resulted in a fast, reproducible, and convenient method for extracting bioactive compounds at low temperature. Total PC, TAA and, caffeine, chlorogenic acid, caffeic acid, theobromine, and kaempferol concentrations determined in the extracts obtained by the ultrasound-assisted extraction of YM leaves varied depending on the different industrial processing steps. PCA showed that the lowest PC, TAA by DPPH, caffeine, theobromine, chlorogenic acid, caffeic acid, and kaempferol content were determined for *green* YM, suggesting that YM exposure to high temperatures that had occurred during the drying and aging processing stages favored the release of these bioactive compounds.

Additionally, these results demonstrated that the YME obtained from *aged-canchada* leaves exhibited antimicrobial activity in the model media and the CCS juice against relevant subrogate bacteria indicating that the bioactive compounds responsible for this effect were highly stable along processing of yerba mate leaves. As expected, even the addition of *aged-canchada* YM at a low concentration (4 mg/ml) to the juice blend significantly increased the antioxidant activity of the CCS juice blend, as the MBC value was very high to be used in the juice blend. The TEM micrographs revealed that the ultrasound-assisted *aged-canchada* YME induced severe cell damage, significantly greater than that observed in cells exposed to YME obtained by thermal extraction. The most significant changes that differentiated the effect of both extracts on *E. coli* cells were related to the direct rupture of outer structures with efflux of inner cell content that took place in most of the cells exposed to the ultrasound-assisted YME.

In conclusion, the *aged-canchada* YME obtained in this study may be added to different food products and beverages with the main purpose of increasing the concentration of bioactive compounds, thus contributing to boost human body defenses as well as to the overall antimicrobial activity in the food product, by total or partial replacement of other synthetic antimicrobials. In addition, this extract has a significant potential as a plant-derived additive, thus meeting the expectations of those adventurous consumers interested in exploring bolder and novel flavors. Further research, including in vivo studies, is needed to increase the information regarding its potentially beneficial effects, thus analyzing its contribution to the diary polyphenols and antioxidant human intake.

ACKNOWLEDGMENTS

The authors acknowledge the financial support for the research, authorship, and/or publication of this article. This work was supported by Universidad de Buenos Aires (2017-2020 N° 20020160100056BA Project), Instituto Nacional de la Yerba Mate (INYM) (N° 69, 310/2017), and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (2015-0401 Project) of Argentina and from Banco Interamericano de Desarrollo (BID).

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Investigation; Methodology; Writing-review & editing: Marcela Schenk. *Investigation; Writing-review & editing*: Mariana Ferrario. *Resources*: Miguel Eduardo Schmalko. *Investigation*: Roy Rivero. *Resources*: Irene Taravini. *Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing-review & editing*: Sandra Guerrero.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restriction.

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How to cite this article: Schenk M, Ferrario M, Schmalko M, Rivero R, Taravini I, Guerrero S. Development of extracts obtained from yerba mate leaves with different industrial processing steps: Antimicrobial capacity, antioxidant properties, and induced damage. *J Food Process Preserv*. 2021;00:e15482. https://doi.org/10.1111/jfpp.15482